

Into the deep: New discoveries at the base of the green plant phylogeny

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Recent data have provided evidence for an unrecognised ancient lineage of green plants that persists in marine deep-water environments. The green plants are a major group of photosynthetic eukaryotes that have played a prominent role in the global ecosystem for millions of years. A schism early in their evolution gave rise to two major lineages, one of which diversified in the world's oceans and gave rise to a large diversity of marine and freshwater green algae (Chlorophyta) while the other gave rise to a diverse array of freshwater green algae and the land plants (Streptophyta). It is generally believed that the earliest-diverging Chlorophyta were motile planktonic unicellular organisms, but the discovery of an ancient group of deep-water seaweeds has challenged our understanding of the basal branches of the green plant phylogeny. In this review, we discuss current insights into the origin and diversification of the green plant lineage.

Keywords:

■ green algae; Palmophyllales; phylogeny; prasinophytes; Viridiplantae

A brief history of green plant evolution

Green plants are one of the most dominant groups of primary producers on earth. They include the green algae and the embryophytes, which are generally known as the land plants. While green algae are ubiquitous in the world's oceans and freshwater ecosystems, land plants are major structural components of terrestrial ecosystems [1, 2]. The green plant lineage is ancient, probably over a billion years old [3, 4], and intricate evolutionary trajectories underlie its present taxonomic and ecological diversity.

Green plants originated following an endosymbiotic event, where a heterotrophic eukaryotic cell engulfed a photosynthetic cyanobacterium-like prokaryote that became stably integrated and eventually evolved into a membrane-bound organelle, the plastid [5, 6]. This single event marked the origin of oxygenic photosynthesis in eukaryotes and gave rise to three autotrophic lineages with primary plastids: the green plants, the red algae and the glaucophytes. From this starting point, photosynthesis spread widely among the eukaryotes via secondary endosymbiotic events that involved the capture of either green or red algae by diverse non-photosynthetic eukaryotes, thus transferring the captured cyanobacterial endosymbionts (i.e. the plastids) laterally among eukaryotes [5]. Some of these secondary endosymbiotic partnerships have, in turn, been captured by other eukaryotes, known as tertiary endosymbiosis, resulting in an intricate history of plastid acquisition (reviewed in refs. [5–7]). Three groups of photosynthetic eukaryotes have plastids derived from a green algal endosymbiont: the chlorarachniophytes, a small group of mixotrophic algae from tropical seas; the euglenophytes, which are especially common in freshwater systems and some green dinoflagellates. A much wider diversity of photosynthetic eukaryotes, including the dinoflagellates, haptophytes, cryptophytes, chrysophytes, diatoms and brown seaweeds, have obtained plastids from a red algal ancestor either by a single or by repeated endosymbiotic events [6, 8].

An early split in the evolution of green plants gave rise to its two principal lineages, which have subsequently followed radically different evolutionary trajectories (Fig. 1) [1, 9, 10]. One lineage, the Chlorophyta, diversified as plankton in the

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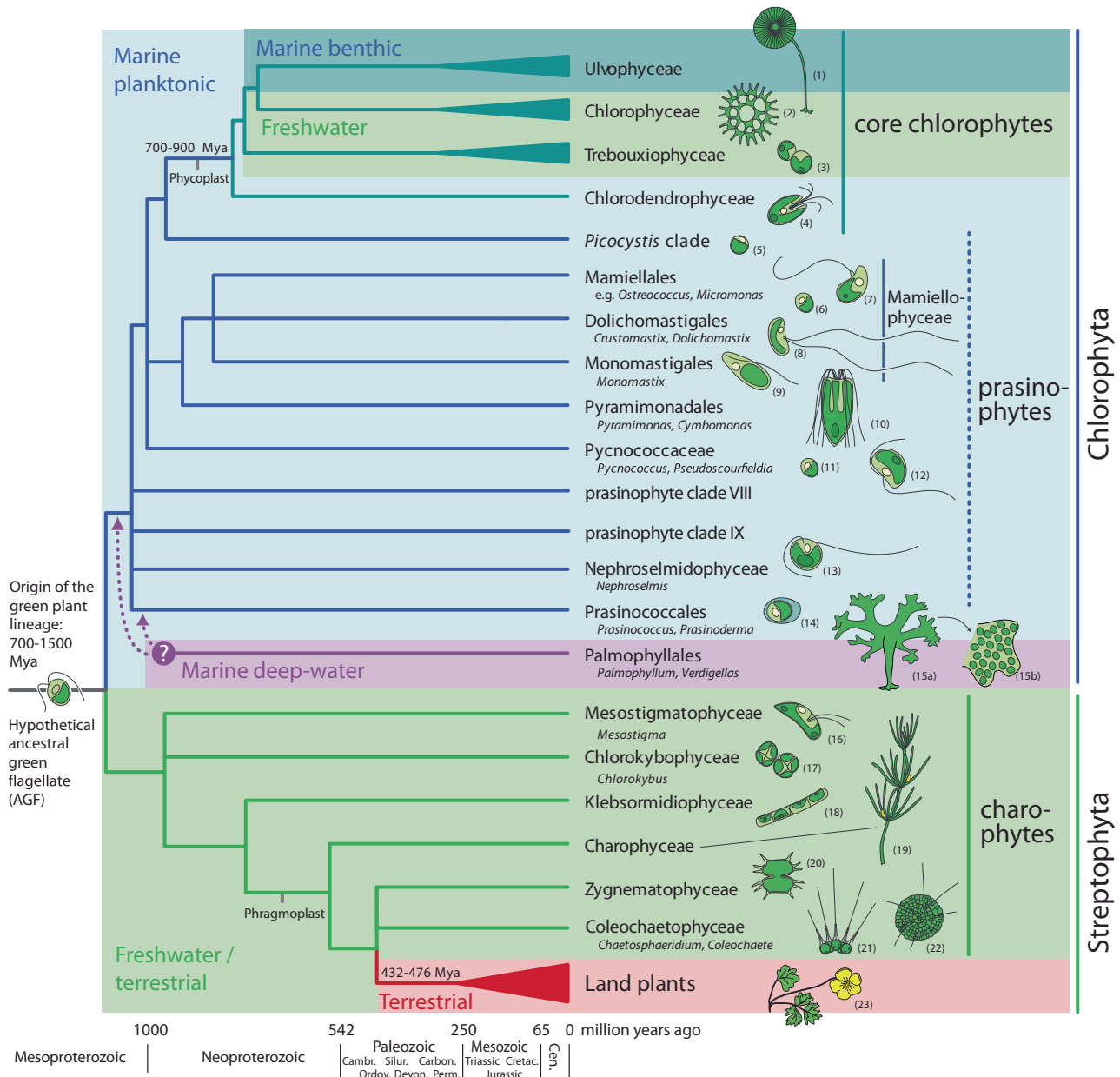


Figure 1. Phylogenetic relationships among the main lineages of green plants. The tree topology is a composite of accepted relationships based on molecular phylogenetic evidence [1, 9–11, 22, 23, 35, 36, 56, 71, 78]. Uncertain phylogenetic relationships are indicated by polytomies. The divergence times are rough approximations based on the fossil record and molecular clock estimates [2–4, 13, 14]. These age estimates should be interpreted with care as different molecular clock studies have shown variation in divergence times between major green plant lineages. Drawings illustrate representatives of each lineage: (1) *Acetabularia*, (2) *Pediastrum*, (3) *Chlorella*, (4) *Tetraselmis*, (5) *Picocystis*, (6) *Ostreococcus*, (7) *Micromonas*, (8) *Crustomastix*, (9) *Monomastix*, (10) *Pyramimonas*, (11) *Pycnococcus*, (12) *Pseudoscourfieldia*, (13) *Nephroselmis*, (14) *Prasinococcus*, (15) *Verdigellas* (a: general habit, b: individual cells in a gelatinous matrix), (16) *Mesostigma*, (17) *Chlorokybus*, (18) *Klebsormidium*, (19) *Chara*, (20) *Xanthidium*, (21) *Chaetosphaeridium*, (22) *Coleochaete*, (23) *Ranunculus*.

oceans and gave rise to the modern prasinophytes and the core chlorophytes that radiated in marine coastal and freshwater environments. The Chlorophyta now encompass a large diversity of green algae with a bewildering variety of body forms,

eco-physiological traits and life cycle strategies [1]. The second lineage, the Streptophyta, evolved in freshwater and damp terrestrial habitats and colonised dry land approximately 476–432 million years ago, giving rise to the land plants [11]. Contemporary streptophytes comprise a diverse array of mainly freshwater algae (collectively termed the charophytes) and the vastly species-rich land plants [11].

The early evolutionary history of the Chlorophyta in the oceans of the Mesozoic and Neoproterozoic (between 700 and 1,500 million years ago) is marked by a radiation of planktonic unicellular organisms [2]. These ancestral green algae were of fundamental importance to the eukaryotic ‘greening’ that shaped the geochemistry of our planet [12]. Although the fossil

record is clearly incomplete, analysis of microfossils suggests that green algae were prevalent in the eukaryotic oceanic phytoplankton of the Paleozoic era [2, 13, 14]. Subsequently, the red plastid-containing dinoflagellates, coccolithophores and diatoms increased in abundance to largely displace the green algae in the phytoplankton from the end-Permian extinction to the present. This evolutionary transition has been related to a long-term change in the chemistry of the ocean during the Mesozoic, combined with specific eco-physiological traits of the red plastid-containing lineages [15]. Trace element usage in algae with a red-type plastid differs from that of green algae, which may have been advantageous following a shift in the redox conditions of the oceans [16]. The pigment sets of red plastids provide for higher underwater photosynthetic efficiency compared to green plastids and may be another explanation for the red dominance in the seas [2, 17]. In addition, the success of lineages with red-type plastids has been explained by better portability of red-type plastids via secondary endosymbiosis to diverse eukaryotic hosts [16], although this hypothesis has been questioned [18].

Despite this red dominance in the phytoplankton, green algae continue to play prominent roles in contemporary marine environments. Prasinophytic picoplanktonic species (i.e. with cells smaller than 3 μm) can dominate both photosynthetic biomass and production in open oceans and coastal systems [19]. In addition, the green seaweeds of the class Ulvophyceae, which radiated in marine benthic habitats in the Neoproterozoic [20–22] (Fig. 1), form key components in many contemporary coastal environments.

The first eukaryotic algae in freshwater environments were probably unicellular streptophytes, which prevailed in these ecosystems in the Proterozoic [23]. During the Paleozoic, the two principal multicellular groups of charophytes, the conjugating green algae (Zygnematophyceae) and stoneworts (Charophyceae) radiated, and the latter dominated freshwater macrophytic communities between the Permian and Early Cretaceous [24]. In the Late Cretaceous and Tertiary, they were largely replaced by freshwater angiosperms. Two classes of the Chlorophyta, the Chlorophyceae and Trebouxiophyceae, adapted to freshwater environments during the Neoproterozoic [4] (Fig. 1) and dominated freshwater planktonic assemblages during the Paleozoic and Mesozoic eras while the diversity and abundance of charophytes gradually decreased [23, 24]. The demise of green algal dominance of freshwater phytoplankton began with the appearance of freshwater dinoflagellates in the Early Cretaceous, and the radiation of diatoms and chrysophytes during the Cenozoic.

The dominance of algae with red-type plastids in the seas (and to a lesser extent in freshwater environments) is in sharp contrast to the situation on land, where photosynthesis has been dominated by the green land plants ever since they colonised the terrestrial environment in the Ordovician [25].

Deep branches of the Chlorophyta

Molecular phylogenetic, ultra-structural and biochemical studies have identified the prasinophytes as a paraphyletic assemblage of free-living unicellular organisms with a wide variety of cell shapes (Fig. 1), flagellar numbers and behaviour,

body scale shapes, mitotic processes, biochemical features and photosynthetic pigment signatures [26–30].

The critical phylogenetic position of the prasinophytes, diverging early from the remaining Chlorophyta (Fig. 1), reinforced the notion that the ancestral chlorophytes were marine planktonic unicellular flagellates with characters typical of extant prasinophytes, such as the presence of organic body scales [31, 32]. The nature of this hypothetical ancestral green flagellate (AGF), however, still remains uncertain. Moestrup [33] proposed that small, simple flagellate cells most closely resemble the AGF. Other researchers have interpreted the food-uptake mechanism of some complex flagellates as a character inherited from a phagotrophic ancestor of the green plants [2, 34, 35].

A better understanding of prasinophytic diversity and relationships is crucial to elucidate the nature of the common ancestor of green plants. Originally, only flagellate unicellular organisms covered with organic body scales were classified in the prasinophytes [31]. The discovery of several new species and the application of environmental sequencing have revealed greater morphological and ecological diversity [28, 36, 37]. Non-motile (coccolithoid) forms have been identified in several of the major prasinophytic lineages and many members lack scales or have other types of cell coverings (Table 1). Prasinophytes are primarily marine, but several representatives have adapted to freshwater environments.

Although there is little doubt that sex pre-dates diversification of extant eukaryotes [38, 39], it has rarely been observed in prasinophytes. A notable exception is *Nephroselmis*, where sexual reproduction has been detected in cultures [40, 41]. However, circumstantial evidence points towards a much wider occurrence of sex among prasinophytes. For example, members of the Pyramimonadales produce walled cysts that contain two chloroplasts, suggestive of gamete fusion [34]. In addition, sexual reproduction has been implied in *Ostreococcus* and *Micromonas* based on the occurrence of sex-related and meiosis-specific genes in their genomes [12, 42].

Several studies have aimed at resolving the relationships among the prasinophytic lineages, which has proven to be a difficult task due to the antiquity of these divergences. Small subunit nuclear ribosomal DNA (18S rDNA) sequences have long been the main source of data for phylogenetic inference within the green plant lineages [43]. Although 18S data have been useful in delineating the main prasinophytic lineages [27, 30, 36], analyses of these single gene datasets have not resolved the relationships among them. A robust phylogeny for an ancient lineage, such as that of green plants, requires analysis of a large number of genes.

Multi-gene data derived from chloroplast genomes, which are presently available for five prasinophytes, are just beginning to shed light on the ancient divergences of the Chlorophyta. A recent chloroplast phylogenomic analysis identified *Nephroselmis* (Nephroselmidophyceae) as the earliest-branching chlorophytic lineage [35] (Fig. 1). This flagellate with a complex covering of scales and two unequal flagella (Fig. 2A and B, Table 1) might thus represent our best guess of what the AGF might have looked like. Interestingly, *Nephroselmis* is one of the few prasinophytes in which sexual reproduction has been well documented [41].

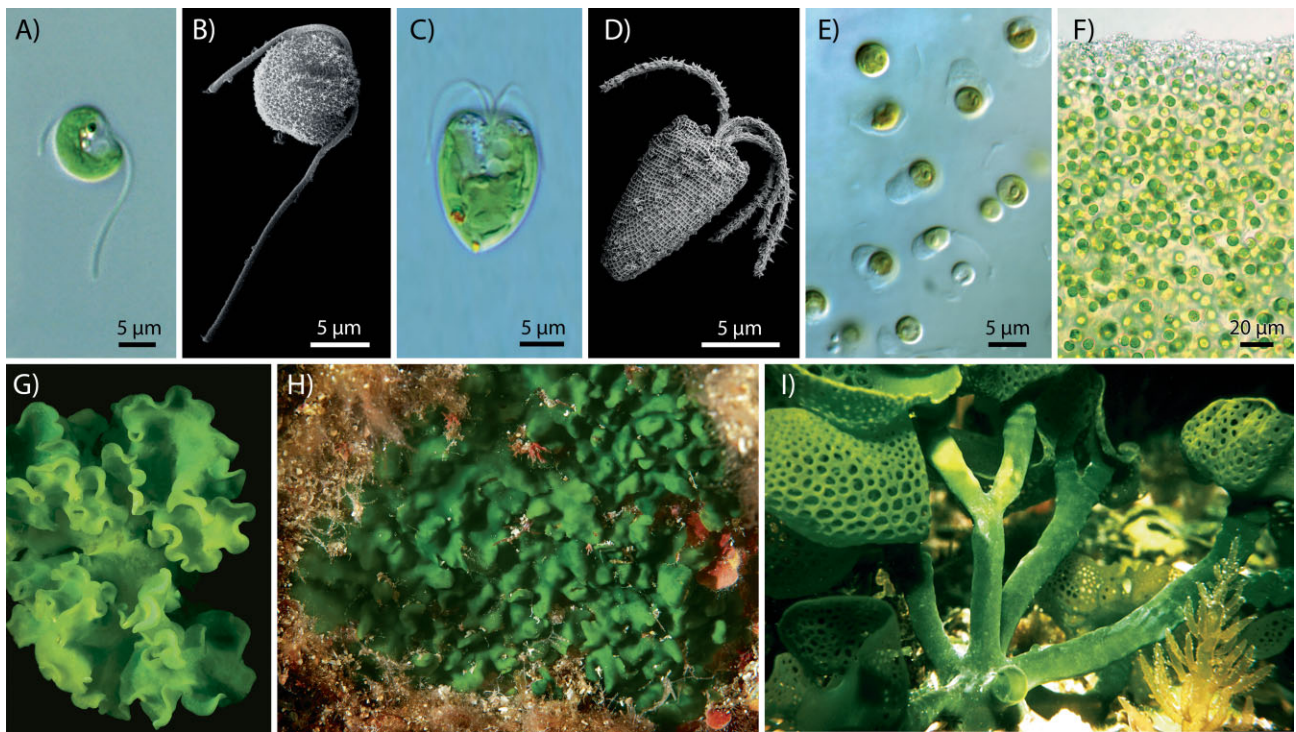


Figure 2. Representatives of some early-diverging lineages of the Chlorophyta. **A:** Light micrograph of *Nephroselmis* (Nephroselmidophyceae) showing two laterally inserted, unequal flagella and a cup-shaped chloroplast (image courtesy of William Bourland). **B:** Scanning electron micrograph of *Nephroselmis* showing a complex covering of organic body scales (image courtesy of Shoichiro Suda, University of the Ryukyus). **C:** Light micrograph of *Pyramimonas* (Pyramimonadales) showing four similar flagella emerging from an anterior depression, a large cup-shaped chloroplast and eyespot located in the lower left (image courtesy of David Patterson and Bob Andersen, Provasoli-Guillard National Center for Culture of Marine Phytoplankton). **D:** Scanning electron micrograph of *Pyramimonas* showing body and flagella covered with different types of scales (image courtesy of Rick van den Enden, Australian Antarctic Division[©] Commonwealth of Australia). **E:** Light micrograph of *Prasinococcus* (Prasinococcales) showing non-motile unicellular organisms, each embedded in gelatinous capsules (image courtesy of Daniel Vaultot, Station Biologique de Roscoff). **F:** Cross-section of a *Palmophyllum* plant (Palmophyllales), composed of coccoid cells embedded in a gelatinous matrix. **G:** *Verdigellas*, a member of the Palmophyllales growing in deep-water habitats (100–200 m) of the western Atlantic Ocean, attaches to the substrate by a central holdfast structure (not visible) above which the rest of the body expands (image courtesy of Mark and Diane Littler, National Museum of Natural History, Smithsonian Institution). **H:** *Palmophyllum*, forms irregularly lobed crusts that are tightly fixed to the substrate and, in the Mediterranean Sea, grows down to depths of 130 m (image courtesy of Véronique Lamare, Données d'Observations pour la Reconnaissance et l'Identification de la faune et de la flore Subaquatiques). **I:** *Palmoclathrus*, a genus from deep-water habitats (to depths of 60 m) of Southern Australia, characterised by perennial stalks from which seasonal, net-like blades grow (photograph by Kevin Branden, Board of the Botanic Gardens & State Herbarium[©], Adelaide, South Australia).

The close relationship between the Pyramimonadales and the Mamiellophyceae was an unexpected result from chloroplast phylogenomic studies [35] (Fig. 1). The Pyramimonadales are relatively large flagellates with complex body scale coverings (Fig. 2C–D), and, as mentioned above, some of its members are unique among green plants in possessing a food uptake apparatus [34]. The Mamiellophyceae is a large group comprising the morphologically and ecologically diverse Mamiellales and two smaller clades, the

Monomastigales and Dolichomastigales [36]. The phylogenetic affinity of the latter two has long been uncertain because several of their members lack scales and have atypical surface structures (Table 1). The Mamiellales are probably the largest and most diverse group of prasinophytes (Table 1). Several members (e.g. *Ostreococcus* and *Micromonas*) may form major components of marine picoeukaryotic communities [19, 44, 45]. These algae have cell sizes smaller than those of many bacteria and show highly reduced cellular complexity and unusually compact genomes [12, 42, 46]. These minute unicellular organisms have been regarded as ‘the bare limits of life as a free-living photosynthetic eukaryote’ [42] and likely evolved through secondary reduction from larger and more complex flagellates [35].

There are several other groups of early-branching prasinophytes that we cannot place in the phylogenetic tree with any great precision, either because only single-gene data are available or because

genome-scale phylogenetic analyses generate equivocal results.

1. The Pycnococcaceae is a small clade of marine flagellates and coccoids (Fig. 1, Table 1). Some studies based on 18S rDNA sequences have related this clade with the Nephroselmidophyceae [27, 30], but this relationship has not been supported by chloroplast multi-gene analyses [35].
2. The Prasinococcales includes a few marine coccoid prasinophytes [47, 48] (Fig. 2E, Table 1) and has been suggested to

Table 1. Characteristics of the major prasinophytic lineages

Lineage and members	Morphology and life cycle	Ecology
Picocystis clade: <i>Picocystis</i> ⁽⁵⁾ and several undescribed taxa	Scale-less coccoids surrounded by a thin cell wall [94]. Sexual reproduction unknown	Picoplanktonic communities in saline lakes (<i>Picocystis</i>) or oceans [50, 94]
Mamiellophyceae – Mamiellales: <i>Ostreococcus</i> ⁽⁶⁾ , <i>Bathycoccus</i> , <i>Micromonas</i> ⁽⁷⁾ , <i>Mantoniella</i> , <i>Mamiella</i>	Structurally simple, wall-less unicellular organisms, including scaly coccoids (<i>Bathycoccus</i>), naked coccoids (<i>Ostreococcus</i>), naked uniflagellates (<i>Micromonas</i>) and scaly biflagellates (<i>Mantoniella</i> , <i>Mamiella</i>) [36]. Scales (when present) with a typical spider-web pattern [32]. <i>Micromonas</i> and <i>Mantoniella</i> with palmelloid phase in the life cycle [32]. Indirect evidence for sexual reproduction from genomic data [12, 42]	Marine planktonic. <i>Ostreococcus</i> and <i>Micromonas</i> can form major components of picoeukaryotic communities [19, 44, 45]
Mamiellophyceae – Dolichomastigales: <i>Crustomastix</i> ⁽⁸⁾ , <i>Dolichomastix</i>	Biflagellates with cells covered with spider-web or circular-patterned scales (<i>Dolichomastix</i>) [28, 32] or cells scale-less and covered with a thin, double-layered membrane (<i>Crustomastix</i>) [28, 95]. Sexual reproduction unknown	Mainly marine planktonic; a few species from freshwater environments [36]
Mamiellophyceae – Monomastigales: <i>Monomastix</i> ⁽⁹⁾	Flagellates with a single mature flagellum (second flagellum present as a basal body only), cells covered with very thin imbricate scales, resembling those of chrysophytes and prymnesiophytes [33]. Only known to reproduce asexually, involving cyst formation	Freshwater habitats [36]
Pyramimonadales: <i>Pyramimonas</i> ⁽¹⁰⁾ , <i>Cymbomonas</i> , <i>Halosphaera</i> , <i>Pterosperma</i> , <i>Prasinopapilla</i>	Large flagellates, generally with four (sometimes 8 or 16) flagella, covered with diverse and complex body scales in multiple layers [31, 32]. Some mixotrophic species of <i>Cymbomonas</i> and <i>Pyramimonas</i> possess a food uptake apparatus [34, 96]. Indirect evidence for sexual reproduction from resistant cysts containing two chloroplasts [34]. Some <i>Pyramimonas</i> species with a palmelloid phase in the life cycle [32]	Marine and freshwater habitats [32]
Pycnococaceae: <i>Pycnococcus</i> ⁽¹¹⁾ , <i>Pseudoscourfieldia</i> ⁽¹²⁾	Scale-less coccoids surrounded by a thin cell wall (<i>Pycnococcus</i>) [32] or wall-less flagellates with two unequal flagella, surrounded with simple scales (<i>Pseudoscourfieldia</i>) [32, 97]. Culture observations and sequence data indicate that both morphologies may represent different phases of the life cycle [28, 30, 32, 98]	Marine picoplanktonic communities [32, 97]
Nephroselmidophyceae: <i>Nephroselmis</i> ⁽¹³⁾	Relatively large, asymmetrical cells with a complex covering of diverse scales in multiple layers [32], and two laterally inserted, unequal and heterodynamic flagella [97]. Sexual reproduction detected in culture [40, 41]	Marine and freshwater environments [99]
Prasinococcales: <i>Prasinococcus</i> ⁽¹⁴⁾ , <i>Prasinoderma</i>	Small, scaleless coccoids with thick cell walls [37]. Cells of <i>Prasinococcus</i> are embedded in gelatinous capsules, secreted by complex pores ('Golgi-decapore complex') [48]. Only known to reproduce asexually	Marine habitats [37, 48]
'Clade VIII'	Known from environmental sequencing only	Marine picoplanktonic communities [51, 52]
'Clade IX'	Known from environmental sequencing only	Marine picoplanktonic communities [50, 51]

Numbers in round brackets refer to the drawings of the organisms in Fig. 1.

- form an early-diverging clade based on 18S data [30] (Fig. 1). Multi-gene data has not yet been generated for this group.
- The *Picocystis* clade has been identified by environmental and culture-based sequencing. It includes a number of undescribed coccoid prasinophytes, along with the saline lake-dwelling coccoid *Picocystis* (Table 1). 18S and multi-gene phylogenies have allied this clade with the core chlorophytes (Fig. 1), but support for this relationship is not strong [30, 36, 49].
 - Environmental sequencing of photosynthetic picoeukaryotic communities has identified two additional prasinophytic clades with uncertain affinities (termed clades VIII and

IX) [50–52]. As these organisms are only known from DNA sequence data, nothing is known about their morphology.

One of the ancestral prasinophytic lineages has given rise to the ecologically and morphologically diverse core chlorophytes (Fig. 1). This group includes the early-diverging Chlorodendrophyceae, a small clade of marine and freshwater quadriflagellates [30]. The three other clades are more diverse and comprise unicellular as well as multicellular organisms. The core chlorophytes are characterised by a new mode of cell division that is mediated by a phycoplast, which was subsequently lost in the Ulvophyceae [1]. Several eco-physiologi-

cal adaptations have allowed successful radiation of the Trebouxiophyceae and Chlorophyceae in freshwater and terrestrial habitats. The Ulvophyceae, which are best known as the green seaweeds, have mainly diversified along marine shorelines where they frequently dominate rocky shores and tropical lagoons. This clade has evolved an unequalled diversity of body forms, ranging from microscopic unicellular organisms to multicellular or giant-celled algae with unique cytological and physiological features [22]. Several members of the core chlorophytes live in symbiosis with various eukaryotic organisms, including fungi to form lichens, ciliates, cnidarians, foraminifera and vertebrates [53–55].

An ancient lineage of deep-water green seaweeds

A recently published study has provided evidence for another early-diverging chlorophytic lineage, the Palmophyllales [56]. This group includes the little-known benthic seaweeds *Palmophyllum*, *Verdigellas* and possibly *Palmoclathrus*; three genera from marine deep-water and other dimly lit environments. Although gene sequence-based phylogenies support a deeply branching Palmophyllales group, its exact phylogenetic placement remains uncertain. Analyses of the plastid genes *rbcL* and *atpB* placed the Palmophyllales sister to the remaining Chlorophyta. However, analysis of nuclear 18S rDNA sequences allied the Palmophyllales with the early-diverging Prasinococcales (Fig. 1). The latter relationship is supported by some shared cytological characteristics, such as a mucus-secreting system [48, 57] and similarities in cell division [37, 47, 58].

Members of the Palmophyllales are characterised by a unique type of multicellularity. They form well-defined macroscopic bodies composed of small spherical cells embedded in a firm gelatinous matrix (palmelloid organisation) [57, 59–61]. Although the cells are separated and undifferentiated (Fig. 2F), several Palmophyllales have evolved large, complex erect bodies. For example, species of *Verdigellas* (Figs. 1 and 2G) attach to the substrate by means of a holdfast structure above which the rest of the body expands, resulting in umbrella-like plants that are well-adapted to capture the dim light in deep-water habitats. *Palmoclathrus*, a genus from temperate waters, are characterised by perennial stalks from which seasonal, net-like blades grow [59] (Fig. 2I). *Palmophyllum* is morphologically simpler, forming irregular lobed crusts that are tightly attached to the substrate (Fig. 2H). Despite careful investigation, motile stages or ultra-structural traces of flagella have never been observed [57, 58, 60]. Interestingly, a number of prasinophytes have been described to have palmelloid stages in their life cycle, although they never form large and complex bodies like the Palmophyllales (Table 1). The early-diverging nature of the non-flagellate Palmophyllales and Prasinococcales, along with the wide phylogenetic distribution of non-motile prasinophytes, raises questions about the nature of the green plant ancestor. Although there is little doubt that flagella must have been present in a life cycle stage of the green plant ancestor, it may be possible that this ancestor was a non-motile unicellular organism with transient motile stages.

It is remarkable that an ancient lineage like the Palmophyllales is restricted to deep-water or other dimly lit habitats. Low-light ecosystems present a challenging environment for photosynthetic organisms and relatively few algae live in such habitats [62]. *Verdigellas* has been recorded from depths down to 200 m [56, 61], where only about 0.05% of the irradiance at the water surface remains [62]. This results in extremely low primary productivity in *Verdigellas* compared to shallow-water green seaweeds [63]. *Palmophyllum* and *Palmoclathrus* species generally grow at depths of between 40 and 100 m [59, 60]. *Palmophyllum* is also found in shallower, shady areas like crevices and under rock overhangs [60].

Members of the Palmophyllales lack the green light-harvesting photosynthetic pigments siphonoxanthin and siphonoin, which are found in several low-light adapted green algae [58, 60]. Instead, they maintain high concentrations of chlorophyll b, which absorbs the blue-green light of deeper water more efficiently than does chlorophyll a [64].

The ability to grow in deep, low-light habitats may be of key importance to the persistence of Palmophyllales. Compared to shallow habitats, deep-water environments are characterised by diminished abiotic stressors (e.g. wave action and temperature variation) and reduced grazing and competition for substrate. Whereas the more recently evolved green seaweeds (Ulvophyceae) of the core chlorophytes possess morphological and biochemical adaptations that allow them to withstand such stresses [65], the Palmophyllales lack protective attributes such as calcification or cortication, and they may have found refuge from competition and herbivory in deep-water habitats [56].

Marine deep-water environments are home to phylogenetic relicts of other lineages of organisms such as the hagfishes [66], chimaeras and cow sharks [67], stalked crinoids and other invertebrates [68]. The onshore-offshore hypothesis posits the shallow-water origination and deep-water retreat of marine lineages in the fossil record [69]. The early-branching position of the species-poor, deep-water Palmophyllales as compared to the diverse and predominantly shallow-water prasinophytes and core chlorophytes may be interpreted as an example of this phenomenon in photosynthetic organisms [56].

Ancient streptophytes and the progenitors of land plants

The origin of land plants was a key event in the history of life and has led to important changes in the earth's environment, including the development of the entire terrestrial ecosystem [25]. Many studies have focused on the relationship among charophytes and have sought to determine the origins of land plants [9, 10, 70–72].

The charophytes are mostly freshwater green algae with diverse morphologies ranging from simple unicellular and filamentous organisms to complex and highly specialised macrophytes. Morphological and molecular data have revealed six distinct groups of charophytes: Mesostigmatophyceae, Chlorokybophyceae, Klebsormidiophyceae, Zygnematophyceae, Charophyceae, and Coleochaetophyceae [11] (Fig. 1). Phylogenetic analyses of multi-gene datasets have clarified

Glossary

Biflagellate: Having two flagella.

Body scales: Organic (non-mineralised) structures, produced within the Golgi apparatus, and covering the cell surface of many prasinophytic species. Prasinophytic body scales are remarkably diverse, including plate-like, hair-like and complex three-dimensional structures [31, 32].

Coccolid: Spherical, non-motile unicellular microorganism.

Flagella: Long whip-like organelles that propel cells through a liquid medium. Flagella contain a highly conserved (9 + 2) arrangement of microtubules. They are homologous with cilia, but generally longer and less numerous.

Flagellate: *Noun:* Motile unicellular eukaryotic microorganism that swims by means of flagella. Flagellates include photosynthetic and heterotrophic species that do not form a natural group of organisms, but are distributed in several distantly related eukaryotic groups. *Adjective:* bearing one or more flagella.

Mixotrophic: Having partly autotrophic and partly heterotrophic nutrition.

Palmelloid: A type of algal body organisation, with cells that are separate but remain enclosed within a mucilage envelope.

Paraphyletic group: A group of organisms that has evolved from a common ancestor but does not contain all descendants of that ancestor. Green algae and charophytes are paraphyletic groups because they do not include land plants. Similarly, prasinophytes are paraphyletic with the exclusion of the core chlorophytes. Paraphyletic groups are characterised by shared primitive

(plesiomorphic) characters. For the green algae, these include the presence of double membrane-bound plastids containing chlorophyll a and b, and several ultra-structural features of the chloroplast and flagella, all of which are also shared with land plants.

Phagotroph: Heterotrophic or mixotrophic organism that ingests nutrients by engulfing solid particles.

Phragmoplast: Array of microtubules oriented perpendicularly to the plane of cell division, determining the formation of the cell plate and new cell wall. Phragmoplasts occur in land plants and their closest charophytic relatives, the Charophyceae, Zygnemato-phyceae and Coleochaetophyceae.

Phycoplast: Array of microtubules oriented parallel to the plane of cell division, determining the formation of a new cell wall. Phycoplasts occur in the core chlorophytic classes, i.e. the Chlorodendrophyceae, Trebouxiophyceae and Chlorophyceae.

Picoplanktonic: The fraction of plankton comprising cells of between 0.2 and 3.0 μm .

Plasmodesmata: Cytoplasmic threads running transversely through cell walls that connect the cytoplasm of adjacent cells.

Quadriflagellate: Having four flagella.

Red-type plastid: Plastids derived from red alga arising from secondary or tertiary endosymbiosis.

Siphonin and siphonoxanthin: Xanthophyll accessory pigments found in Ulvophyceae and some prasinophytes. The possession of these pigments is believed to be an adaptation to life in deep water, because they are well suited to harvesting of the green light that penetrates to these depths [64].

Uniflagellate: Having a single flagellum.

the relationships among these lineages, although some important questions remain [9, 10, 70–74].

Molecular phylogenies have provided evidence that the morphologically simple charophytes *Mesostigma* (Mesostigmatophyceae) and *Chlorokybus* (Chlorokybophyceae) form the earliest-diverging streptophytic lineages (Fig. 1) [9, 10, 71, 74]. This result is consistent with ultra-structural features of their cells [1, 32] and discrete molecular characteristics, such as shared multi-gene families or gene duplications [75, 76]. Some phylogenies inferred from nuclear multi-gene data have placed *Mesostigma* as sister group to the remaining Streptophyta [22, 71]; a position that is supported by the fact that *Mesostigma* is the only streptophyte with a motile vegetative stage – a presumed ancestral feature of green plants. Conversely, phylogenies based on complete chloroplast genomes have suggested a sister relationship between *Mesostigma* and *Chlorokybus* [9, 10]. *Mesostigma* is a freshwater biflagellate unicellular organism with a unique suite of photosynthetic pigments. Like many prasinophytes, the cell and flagella are covered with diverse organic scales. *Chlorokybus* is found in moist terrestrial environments where it forms groups of a few non-motile cells [11].

Gene sequence-based phylogenies unambiguously show that the freshwater or terrestrial filamentous Klebsormidiophyceae diverged after the Mesostigmatophyceae and Chlorokybophyceae [70, 71, 77] (Fig. 1); a phylogenetic position that is further supported by several chloroplast genomic features [78].

Interestingly, sexual reproduction has not been observed in any of these early-diverging lineages and is only known in the later-diverging streptophytes [11]. However, determining whether these lineages are truly asexual will require genomic screening, as numerous allegedly asexual chlorophytic members have been shown to have cryptic potential for sex by the presence of meiosis and sex-related genes in their genomes [12, 42, 79].

In contrast to the three early-diverging streptophytic lineages (Mesostigmatophyceae, Chlorokybophyceae and Klebsormidiophyceae) that undergo cell division by furrowing, the cluster consisting of the Charophyceae, Zygnemato-phyceae, Coleochaetophyceae and the land plants evolved a new mechanism of cell-wall formation during cell division, which involved the production of a phragmoplast. In addition, most of the later-diverging streptophytes have cell-walls with plasmodesmata, facilitating cytoplasmic communication between cells and development of complex tissues [80].

Numerous studies have focussed on identifying the closest living relative of land plants, and different charophytes have been suggested based on morphological, biochemical and molecular data [11, 23]. Gene sequence-based phylogenies have been sensitive to taxon and gene sampling and have revealed the morphologically complex Charophyceae [22, 70, 81] or Coleochaetophyceae [35, 71], or the structurally simpler Zygnematophyceae [9, 10, 72, 74] as the sister lineage of the land plants.

The colonisation of dry land involved many challenges such as desiccation, increased temperature fluctuations, exposure to UV radiation and gravity [82–84]. Several physiological and morphological innovations have led to successful adaptation to terrestrial life [23, 80, 82]. Some of these are also found in one or more charophytes and thus likely evolved before the origin of land plants, including cellulosic cell walls, multicellularity, differentiated cells and tissues, intercellular communication networks (plasmodesmata and plant hormones), zygote retention and placenta [23, 80]. Other innovations, such as a sexual life history involving an alternation of two multicellular bodies, and protected embryos appear to be unique to land plants [80]. Additional adaptations to life on dry land included enhanced osmoregulation, desiccation and freezing tolerance, and heat resistance [82, 85].

Comparative genomic studies have indicated that the molecular bases of many land plant innovations evolved before the transition to land [23, 72, 86]. For example, several genes that were thought to be important in the evolution of land plants [80] may have true orthologs with similar function in the Coleochaetophyceae and/or Zygnematophyceae [72, 86]. The diversification of embryophytes and the evolution of complex plants was associated with expansion of numerous gene families, including MADS box genes [87], homeobox genes [88], OPR genes [89] and genes involved in signalling pathways, such as auxin, ABA and cytokinin [85, 86, 90]. Expansion of the glutaredoxins gene family likely resulted in proteins with novel functions, such as in development and the pathogenesis response [91]. The typical life history of land plants possibly evolved through expansion of homeodomain gene networks [87].

Conclusions and prospects

Molecular phylogenetic studies have drastically reshaped our views of green plant evolution [1, 2, 43]. It is now generally accepted that the green plants evolved into two discrete lineages (Fig. 1). One lineage, the Chlorophyta, includes several early-diverging clades of free-living unicellular organisms (the prasinophytes) and the morphologically diverse core chlorophytes. The other lineage, the Streptophyta, comprises the early-branching charophytic green algae and the land plants.

Resolving the relationships between these early-branching clades is crucial to addressing questions about the origin of the green plant lineage and to learn about the evolutionary trajectories responsible for the remarkable diversity of green algae and the emergence of the land plants. It has become clear that to achieve a reliable phylogenetic resolution for ancient groups like the green plants, many genes from many species must be analysed by applying state-of-the-art phylo-

genetic techniques [92, 93]. Multi-gene phylogenetic investigations are just starting to shed light on the basal branches of the green plant phylogeny [9, 10, 35]. High-throughput DNA sequencing techniques can facilitate broader gene and taxon sampling and will undoubtedly lead to more robust phylogenies [71, 72].

The identification of deep-branching lineages is crucial to make inferences about the nature of the common ancestor of the green plant lineage. Sequencing of culture collections and environmental picoplankton samples has led to the discovery of several ancient green algal lineages [27, 30, 36, 50–52]. In addition, sampling from challenging habitats such as marine deep-water ecosystems has recently revealed a previously unrecognized deep-branching lineage of green plants [56]. Further exploration of diversity in under-studied ecosystems such as deep marine waters, tropical coral reefs and sand habitats may lead to the discovery of other ancient groups and further alter our understanding of the early evolution of green plants.

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